

## ORIGINAL RESEARCH

# Characterization of blood pressure and endothelial function in TRPV4-deficient mice with L-NAME- and angiotensin II-induced hypertension

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## Keywords

Endothelium, endothelium-derived hyperpolarizing factor, Hypertension, mesenteric arteries, nitric oxide, TRPV4.

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## Introduction

The endothelium regulates vascular tone by releasing a number of vasodilator factors, including nitric oxide (NO), prostacyclin (PGI<sub>2</sub>), and endothelium-derived hyperpolarizing factors (EDHFs) (Edwards et al. 2010). Various receptor agonists such as acetylcholine (ACh) and bradykinin, as well as mechanical forces (e.g., shear stress), increase endothelial intracellular Ca<sup>2+</sup> concentra-

## Abstract

Transient receptor potential vanilloid type 4 (TRPV4) is an endothelial Ca<sup>2+</sup> entry channel contributing to endothelium-mediated dilation in conduit and resistance arteries. We investigated the role of TRPV4 in the regulation of blood pressure and endothelial function under hypertensive conditions. TRPV4-deficient (TRPV4<sup>-/-</sup>) and wild-type (WT) control mice were given L-NAME (0.5 g/L) in drinking water for 7 days or subcutaneously infused with angiotensin (Ang) II (600 ng/kg per minute) for 14 days, and blood pressure measured by radiotelemetry. TRPV4<sup>-/-</sup> mice had a lower baseline mean arterial pressure (MAP) (12-h daytime MAP, 94 ± 2 vs. 99 ± 2 mmHg in WT controls). L-NAME treatment induced a slightly greater increase in MAP in TRPV4<sup>-/-</sup> mice (day 7, 13 ± 4%) compared to WT controls (6 ± 2%), but Ang II-induced increases in MAP were similar in TRPV4<sup>-/-</sup> and WT mice (day 14, 53 ± 6% and 37 ± 11%, respectively, *P* < 0.05). Chronic infusion of WT mice with Ang II reduced both acetylcholine (ACh)-induced dilation (dilation to 10<sup>-5</sup> mol/L ACh, 71 ± 5% vs. 92 ± 2% of controls) and the TRPV4 agonist GSK1016790A-induced dilation of small mesenteric arteries (10<sup>-8</sup> mol/L GSK1016790A, 14 ± 5% vs. 77 ± 7% of controls). However, Ang II treatment did not affect ACh dilation in TRPV4<sup>-/-</sup> mice. Mechanistically, Ang II did not significantly alter either TRPV4 total protein expression in mesenteric arteries or TRPV4 agonist-induced Ca<sup>2+</sup> response in mesenteric endothelial cells in situ. These results suggest that TRPV4 channels play a minor role in blood pressure regulation in L-NAME- but not Ang II-induced hypertension, but may be importantly involved in Ang II-induced endothelial dysfunction.

tion ([Ca<sup>2+</sup>]<sub>i</sub>) (Nilius and Droogmans 2001) which subsequently leads to the synthesis and release of endothelial vasodilator factors. An increase in endothelial [Ca<sup>2+</sup>]<sub>i</sub> also activates intermediate- and small-conductance Ca<sup>2+</sup>-activated potassium channels (IK<sub>Ca</sub> and SK<sub>Ca</sub>), resulting in endothelium-dependent hyperpolarization (EDH). This hyperpolarization can be directly transmitted to myocytes through myoendothelial gap junctions to induce vasodilation. In addition, K<sup>+</sup> ions

that efflux through endothelial  $K_{Ca}$  channels can induce myocyte hyperpolarization and relaxation by activating smooth muscle, inwardly rectifying  $K^+$  ( $K_{ir}$ ) channels and  $Na^+/K^+$ -ATPases (Edwards et al. 2010).

Accumulating evidence suggests that transient receptor potential (TRP) vanilloid 4 (V4), a member of the TRP superfamily, is an endothelial  $Ca^{2+}$  entry channel involved in NO- and/or EDH-mediated vasodilation in response to flow and receptor agonists in several rodent conduit and resistant arteries (Köhler et al. 2006; Hartmannsgruber et al. 2007; Loot et al. 2008; Earley et al. 2009; Zhang et al. 2009; Mendoza et al. 2010; Adapala et al. 2011; Rath et al. 2012; Sonkusare et al. 2012; Ma et al. 2013). TRPV4 channels are also expressed in endothelial cells of human coronary arterioles (HCAs) and contribute to flow-induced dilation of coronary arterioles from subjects with coronary artery disease (CAD) (Bubolz et al. 2012; Zheng et al. 2013). Of note, this flow-induced dilation of HCAs is mediated by a reactive oxygen species (ROS), namely hydrogen peroxide ( $H_2O_2$ ) that serves as a prominent EDHF in the presence of disease (Miura et al. 2003; Liu et al. 2011; Zhang et al. 2012). Although the TRPV4-mediated dilation has been well-characterized in isolated arteries in vitro, the functional role for TRPV4 in the regulation of vascular tone in vivo remains incompletely understood. Infusion of TRPV4 agonists induced hypotension in anesthetized mice and rats (Willette et al. 2008; Gao et al. 2009; Zhang et al. 2009; Gao and Wang 2010); however, an orally active TRPV4 antagonist did not affect mean arterial pressure (MAP) or heart rate (HR) over 7 days of drug treatment (Thorneloe et al. 2012). In addition, no change in baseline MAP was observed in one line of TRPV4-deficient ( $TRPV4^{-/-}$ ) mice and a trend toward lower MAP in another knockout line (Willette et al. 2008; Earley et al. 2009; Zhang et al. 2009). These studies suggest that TRPV4 may not play a significant role in the basal control of blood pressure. It remains to be determined whether TRPV4 contributes to the regulation of vascular tone in vivo under stress conditions.

This study was designed to further explore the role of TRPV4 in the regulation of blood pressure using conscious  $TRPV4^{-/-}$  mice given two different hypertensive challenges, including  $N^G$ -nitro-L-arginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor, and angiotensin (Ang) II (Ribeiro et al. 1992; Rajagopalan et al. 1996; Mattson 1998; Jung et al. 2003). There is evidence that TRPV4 is more closely linked to EDHF-mediated dilation in small resistance arteries (Köhler et al. 2006; Earley et al. 2009; Zhang et al. 2009; Mendoza et al. 2010; Rath et al. 2012; Sonkusare et al. 2012), and that EDHF may serve as a compensatory mechanism for impaired NO-mediated dilation in the presence of cardiovascular

diseases or risk factors (Félétou and Vanhoutte 2004). As Ang II reduces vascular NO bioavailability and impairs endothelium-dependent dilation via a ROS-mediated mechanism and this endothelial dysfunction has been implicated in various vascular diseases (Rajagopalan et al. 1996; Touyz and Schiffrin 2000; Jung et al. 2003; Garrido and Griendling 2009), we also examined the potential role of TRPV4 in Ang II-induced impairment of endothelium-mediated dilation.

## Methods

### Animals

A total of 55 male wild-type (WT) C57BL/6J and 32 male TRPV4 knockout ( $TRPV4^{-/-}$ ) (Suzuki et al. 2003) mice at 3–4 months of age were used in this study. Mice were housed in our animal facility under a 12/12-h day/night cycle and had access to food and water ad libitum. The study was approved by the Institutional Review Committee. All experiments were conducted in accordance with the Institutional Animals Care and Use Committee guidelines. Genotyping was performed by polymerase chain reaction (PCR) with the following primers: TRPV4 (forward 5'-TGT TCG GGG TGG TTT GGC CAG GAT AT-3' and reverse 5'- GCT GAA CCA AAG GAC ACT TGC ATA G-3'; a 796-bp product in WT and no signal in  $TRPV4^{-/-}$ ), and knockout neomycin cassette (forward 5'- GCT GCA TAC GCT TGA TCC GGC TAC-3' reverse 5'-TAA AGC ACG AGG AAG CGG TCA GCC-3'; a 366-bp product in  $TRPV4^{-/-}$ ).

### Blood pressure measurement

WT and  $TRPV4^{-/-}$  mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) with supplemental anesthesia administered as needed. Using aseptic technique, the catheter tip of a radiotelemetry device (Model TA11PA-C10; Data Sciences International, St. Paul, MN) was inserted into the left carotid artery, and the body of the telemeter implanted subcutaneously in the back of the animal. Animals were maintained at 37–38°C on a heating pad during surgery and recovery from the anesthesia. Antibiotics (Cefazolin, 25 mg/kg, s.c.) and analgesics (Carprofen, 5 mg/kg, s.c.) were administered after surgery. Mice were allowed to recover from implantation for 7 days, and ambulatory MAP, and HR were then measured. Data were collected at 500 Hz for 10 sec every minute and averaged for 12 h light, 12 h dark, and 24 h periods. Baseline parameters were recorded for 3 days prior to drug treatment. L-NAME was added to the drinking water (0.5 g/L) for 7 days, followed by 3-day washout period. For Ang II-induced hypertension, mice were anes-

thetized with 2% isoflurane and an osmotic minipump (micro-osmotic pump model 1002; Alzet, Cupertino, CA) was implanted subcutaneously in the anterior to the mid-scapular region of animals. Ang II was delivered at an infusion rate of 600 ng/kg per minute for 14 days. Some mice received normal saline as sham-operated controls. At the end of *in vivo* study, animals were euthanized with pentobarbital (100 mg/kg, *i.p.*) and mesenteric arteries were dissected for vessel reactivity,  $\text{Ca}^{2+}$  imaging, and protein expression experiments.

### Videomicroscopy

For measurement of vascular reactivity in WT and TRPV4<sup>-/-</sup> mice, first- or second-order branches (100–200  $\mu\text{m}$ ) from the superior mesenteric artery were cannulated with two glass micropipettes for continuous videomicroscopic measurements of diameter as previously described (Mendoza *et al.* 2010). Vessels were pressurized to an intramural pressure of 60 mmHg under no-flow conditions and equilibrated for 1 h at 37°C in Krebs-physiological saline solution (PSS) gassed with 21% O<sub>2</sub> and 5% CO<sub>2</sub>. Vessels were precontracted with U-46619 (10–100 nmol/L) so the internal diameter was ~30–50% of the baseline internal diameter. After the contraction reached steady state, relaxation responses to cumulative concentrations of ACh (10<sup>-9</sup>–10<sup>-5</sup> mol/L), GSK1016790A (10<sup>-9</sup>–10<sup>-7</sup> mol/L), a selective TRPV4 agonist, or sodium nitroprusside (SNP; 10<sup>-10</sup>–10<sup>-5</sup> mol/L) were determined before and after 30-min treatment with L-NAME (100  $\mu\text{mol/L}$ ). To examine the role of smooth muscle hyperpolarization in ACh-induced dilation, arteries were precontracted with high-K (60 mmol/L) Krebs in the presence of L-NAME. At the end of each experiment, papaverine (10<sup>-4</sup> mol/L), an endothelium-independent vasodilator, was added to the vessel bath to determine the maximal dilation for normalization of dilator responses. Vasodilator responses are expressed as percentage of maximal relaxation relative to U-46619 constriction, with 100% representing full relaxation to the maximal diameter.

### Western blotting

Small mesenteric arteries (100–200  $\mu\text{m}$ ) were dissected, cleaned of adipose and connective tissues, and homogenized in an ice-cold lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 0.5% Nonidet P-40, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS]) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany), and centrifuged at 1000g for 10 min at 4°C (Mendoza *et al.* 2010). Protein concentration was determined by using a BCA (bicinchoninic acid)

protein assay kit (Thermo Scientific, Rockford, IL). Protein samples (10  $\mu\text{g}$ ) were separated by SDS-PAGE (polyacrylamide gel electrophoresis) on 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked by 10% milk or BSA for 3 h at room temperature, and incubated overnight at 4°C with appropriate antibodies, including anti-TRPV4 (ACC-034; Alomone Labs, Jerusalem, Israel) or anti- $\beta$ -actin (A5441, clone AC-15; Sigma-Aldrich, St. Louis, MO) antibodies (1:700 in Tris-buffered saline with Tween-20 [TBST] with 2% milk and 1:40,000 in TBST with 2% BSA, respectively) for isolated vessels, or an anti-turbo green fluorescent protein (tGFP) antibody (1:15,000 in TBST with 2% milk, AB513; Evrogen, Moscow, Russia) for TRPV4-transfected endothelial cells (Zheng *et al.* 2013). Blots were then washed with TBST prior to the addition of goat anti-rabbit or goat anti-mouse secondary antibodies (for TRPV4 and GFP, 1:10,000 in TBST with 2% milk;  $\beta$ -actin, 1:20,000 in TBST with 5% BSA) for 2 h at room temperature. Membranes were developed using the ECL Plus reagent (Amersham, GE Healthcare, Pittsburgh, PA). Some membranes were stripped in a stripping buffer solution (Thermo Scientific, Rockford, IL) and reprobed with an anti-eNOS antibody (1:1000 in TBST with 2% milk; cat no. 9572S; Cell Signaling Technology, Danvers, MA) and then with a goat anti-rabbit antibody (1:20,000 dilution in TBST with 2% milk).

### Cell surface protein isolation

Human coronary artery endothelial cells (HCAECs) were obtained from Lonza (Walkersville, MD) and maintained in a full-growth medium (EGM-2MV from Lonza) according to the manufacturer's protocols. To examine the effect of Ang II on the cell surface protein expression of TRPV4, cells at passage six were transduced with recombinant lentiviruses encoding human TRPV4-GFP fusion protein as described previously (Zheng *et al.* 2013). One day after transduction, cells were plated onto 60-mm or 100-mm Petri dishes and grown to 90–95% confluence for additional 2–3 days. Cells were then incubated with Ang II (500 nmol/L) in the full-growth medium for 1 or 24 h. A cell surface protein isolation kit from Pierce was used to isolate plasma membrane proteins from HCAECs, according to the manufacturer's instructions with slight modification. In brief, cells were washed twice with ice-cold phosphate-buffered saline (PBS, Invitrogen, Grand Island, NY) and incubated in PBS containing 0.25 mg/mL sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-Biotin) at 4°C with gentle rocking. After 30 min incubation, a quenching solution was added to stop the biotinylation reaction. Cells were washed three times with ice-cold Tris-

buffered saline (TBS) and then lysed in a lysis buffer (50 mmol/L Tris-HCl [pH 7.40], 150 mmol/L NaCl, 0.5% Nonidet P-40, 1% deoxycholic acid, and 0.1% SDS) supplemented with protease inhibitors. Cell lysates were centrifuged at 12,000g for 10 min at 4°C, and clarified supernatants were taken and assayed for protein concentration by the BCA method (Thermo Scientific). To isolate biotinylated proteins, protein samples (200 µg) were mixed with 50 µL of NeutrAvidin agarose beads (supplied as 50% slurry) and rotated for 3 h at 4°C. The immunoprecipitated proteins were collected by centrifugation at 5000g for 2 min, washed 4–5 times with a wash buffer supplemented with protease inhibitors, and then eluted with 100 µL of 1× Laemmli sample buffer (Bio-Rad, Hercules, CA) with 50 mmol/L dithiothreitol (DTT) by heating for 5 min at 95°C. The eluted proteins (7 µL, equivalent to 15 µg of total protein) and total protein lysates (7 µg) were analyzed by Western blotting as described earlier.

### Measurement of $[Ca^{2+}]_i$

Endothelial  $[Ca^{2+}]_i$  was measured in HCAECs using fura-2 AM, as we described previously (Mendoza et al. 2010; Zheng et al. 2013). HCAECs were plated onto 35-mm glass-bottom petri dishes and grown to 60–70% confluence. Cells were loaded with fura-2 AM (5 µmol/L; Molecular Probes, Grand Island, NY) at room temperature for 30–60 min in a modified Hanks balanced salt solution (HBSS) that contained (in mmol/L): 123 NaCl, 5.4 KCl, 1.6 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 0.4 MgSO<sub>4</sub>, 4.2 NaHCO<sub>3</sub>, 0.3 NaH<sub>2</sub>PO<sub>4</sub>, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 5.5 glucose, and 20 HEPES (pH 7.4 with NaOH). Fluorescence images were captured and analyzed using an image system consisting of an inverted fluorescence microscope (Nikon TE200, Melville, NY) with a 20× fluor objective, a high-speed wavelength switcher (Lambda DG-4 from Sutter Instrument Company, Novato, CA), a PC-controlled digital CCD camera (Hamamatsu C4742-95, Bridgewater, NJ), and Metafluor software (Molecular Devices, Sunnyvale, CA). Fluorescence was continuously recorded for 20–30 min every 3 sec at an emission wavelength of 510 nm with alternative excitation at 340 and 380 nm. Results are presented as the ratio of the fluorescence intensity at 340 versus 380 nm excitation ( $F_{340}/F_{380}$ ). To examine the effect of Ang II on endothelial TRPV4 function, HCAECs were pretreated with Ang II (500 nmol/L in full-growth medium) for 1 or 24 h, and then measured for  $Ca^{2+}$  response to the TRPV4 agonist 4α-PDD (3 µmol/L). Endothelial  $[Ca^{2+}]_i$  was also measured in situ in freshly isolated mesenteric arteries using a modified method we have previously described (Zhang et al. 2009; Mendoza et al. 2010). An arterial segment was cut open along its longitudinal axis and pinned onto a Sylgard-coated dish with lumen side upward. Vessels were incubated with fura-2 AM

(10 µmol/L) at room temperature for 60 min, followed by the  $Ca^{2+}$  assay in Krebs-PSS as described above. The experiments were performed at 37°C unless otherwise indicated.

### Materials and solutions

U46619 was obtained from Cayman Chemical Company (Ann Arbor, MI). GSK1016790A was kindly provided by GlaxoSmithKline Pharmaceuticals. All other chemicals were purchased from Sigma-Aldrich. Stock solutions were made in distilled water, except U46619 (ethanol), GSK1016790A (dimethyl sulfoxide [DMSO]), 4α-PDD (DMSO), and indomethacin (0.2 mol/L Na<sub>2</sub>CO<sub>3</sub>).

### Statistical analysis

All data are presented as mean ± SEM, with *n* indicating the number of animals, vessels, or experimental repeats. For relaxation studies, pEC<sub>50</sub> were calculated. pEC<sub>50</sub> represents the negative logarithm of the concentration of relaxant giving half the maximal relaxation (−logEC<sub>50</sub>); these values were determined directly from individual log concentration–response curves. Comparisons of radiotelemetry results and concentration–response curves of isolated vessels were performed using two-way repeated measures analysis of variance (ANOVA), followed by the Holm–Sidak post hoc test when significance was indicated. For other experiments, significant differences between mean values were evaluated by paired or unpaired *t*-test, or one-way ANOVA followed by the Holm–Sidak multiple comparison test, where appropriate. All procedures were performed using the statistical analysis programs provided in SigmaPlot, version 12. *P* values <0.05 were considered statistically significant.

## Results

### L-NAME-induced hypertension in WT and TRPV4<sup>−/−</sup>

Baseline MAP (12-h daytime) was slightly lower in TRPV4<sup>−/−</sup> (94 ± 2 vs. 99 ± 2 mmHg in WT controls; *n* = 15 and 13, respectively; *P* = 0.047), but baseline HR was similar in TRPV4<sup>−/−</sup> and WT mice (525 ± 12 and 526 ± 8 bpm, respectively). These results are consistent with those of our previous studies showing a trend toward lower blood pressure in TRPV4<sup>−/−</sup> mice using tail-cuff blood pressure measurement and in anesthetized mice using carotid catheters (Mizuno et al. 2003; Zhang et al. 2009).

We next examined whether TRPV4 contributes to blood pressure regulation under conditions with reduced NO-

mediated dilation. L-NAME, a NOS inhibitor, was given to WT and TRPV4<sup>-/-</sup> mice ( $n = 6$  per group) in drinking water (0.5 g/L) for 7 days. L-NAME consumption was similar in TRPV4<sup>-/-</sup> and WT mice ( $2.2 \pm 0.1$  vs.  $2.3 \pm 0.2$  mg/day). 12-h daytime MAP reached its highest point at day 2 following L-NAME administration and then gradually lowered to and maintained at 35–50% of the maximal increase until washout period (Fig. 1A). The MAP values following L-NAME were similar between each group (day 7,  $110 \pm 2$  mmHg in TRPV4<sup>-/-</sup> vs.  $108 \pm 2$  mmHg in WT). However, the percent increase in MAP was slightly higher in TRPV4<sup>-/-</sup> (Fig. 1B; at day 7,  $13 \pm 4\%$  vs.  $6 \pm 2\%$  in WT,  $P < 0.05$ ). HR was reduced in both groups after L-NAME treatment, but the reduction was significantly less in TRPV4<sup>-/-</sup> mice (Fig. 1C). The percent reduction in HR was also less in TRPV4<sup>-/-</sup> mice, but this did not reach statistical significance (Fig. 1D). Both MAP and HR returned to their approximate baseline values 1 day after L-NAME withdrawal.

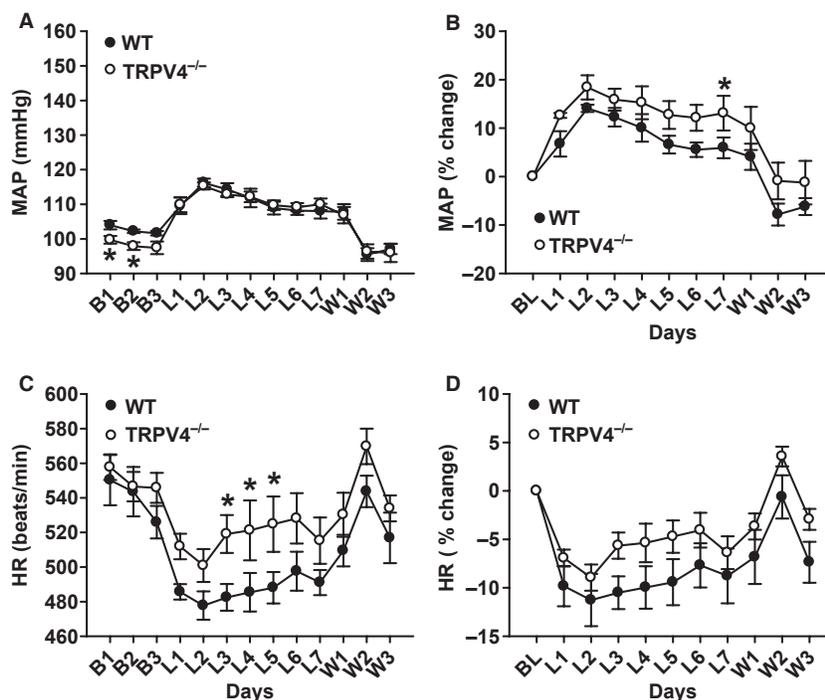
### Ang II-induced hypertension in WT and TRPV4<sup>-/-</sup>

To further test the role of TRPV4 in blood pressure regulation when NO function is reduced, we chronically infused

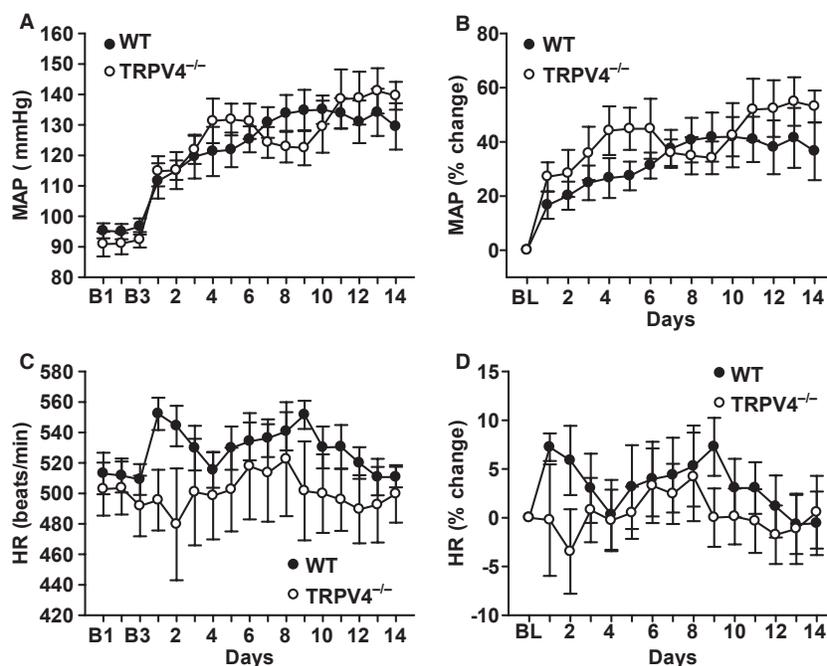
WT and TRPV4<sup>-/-</sup> mice ( $n = 6$  per group) with Ang II using subcutaneous osmotic minipumps (600 ng/kg per min for 14 days). Previous studies have demonstrated that Ang II increases vascular ROS production, and thereby reduces NO bioavailability and endothelium-dependent dilation (Rajagopalan *et al.* 1996; Touyz and Schiffrin 2000; Jung *et al.* 2003; Garrido and Griendling 2009). Infusion of Ang II increased 12-h daytime MAP gradually in both groups (Fig. 2A). The Ang II-induced increases in MAP were similar in TRPV4<sup>-/-</sup> (day 14,  $140 \pm 5$  mmHg) and WT ( $130 \pm 8$  mmHg) mice. The percent increase in MAP tended to be higher (but not statistically significant) in TRPV4<sup>-/-</sup> (Fig. 2B; day 14,  $53 \pm 6\%$ ) compared with WT mice ( $37 \pm 11\%$ ). HR responses to Ang II infusion were similar in TRPV4<sup>-/-</sup> mice and WT controls (Fig. 2C and D). MAP and HR in mice that received normal saline as sham-operated controls at day 14 were not changed compared to baseline values (data not shown).

### Endothelium-dependent dilation in WT and TRPV4<sup>-/-</sup> treated with Ang II

To determine the potential role of TRPV4 in Ang II-induced endothelial dysfunction, small mesenteric arteries were isolated from WT and TRPV4<sup>-/-</sup> mice chronically



**Figure 1.** Mean arterial pressure (MAP) and heart rate (HR) in wild-type (WT) and TRPV4<sup>-/-</sup> mice treated with the nitric oxide synthase inhibitor *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). L-NAME (0.5 g/L) was given in drinking water for 7 days. Ambulatory MAP and HR were recorded by radiotelemetry. Results are presented as 12-h daytime average of MAP (A) and HR (C), and as percent change in MAP (B) and HR (D) relative to baseline values.  $n = 6$  animals for each group. \* $P < 0.05$  vs. WT mice. BL or B, baseline; L, L-NAME; W, washout.

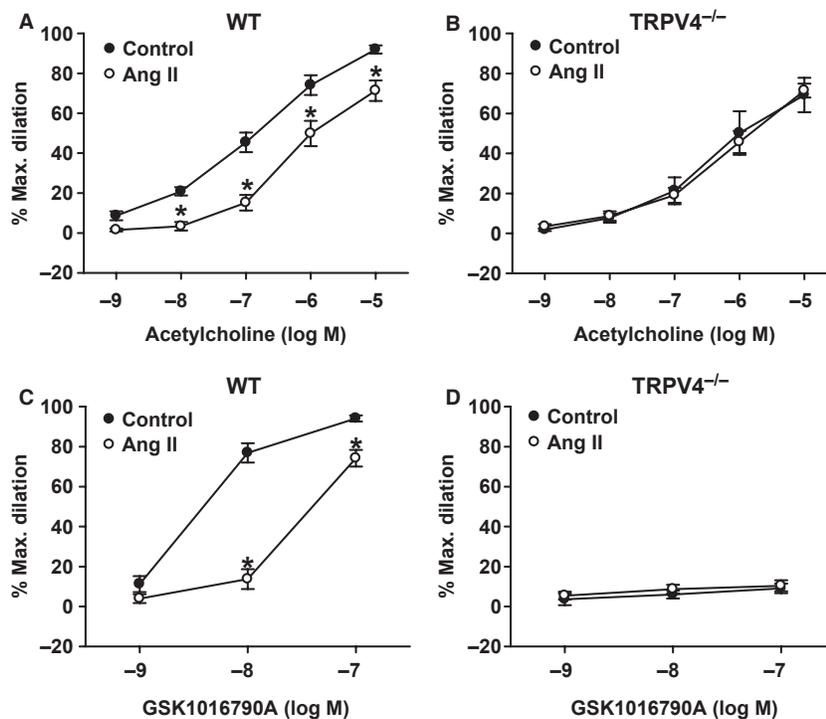


**Figure 2.** Mean arterial pressure (MAP) and heart rate (HR) in wild-type (WT) and TRPV4<sup>-/-</sup> mice treated with angiotensin II. Animals were chronically infused with angiotensin II (600 ng/kg per minute) via subcutaneous osmotic minipumps for 14 days. Ambulatory MAP and HR were recorded by radiotelemetry. Results are presented as 12-h daytime average of MAP (A) and HR (C), and as percent change in MAP (B) and HR (D) relative to baseline values.  $n = 6$  animals for each group. BL or B, baseline.

infused with Ang II (600 ng/kg per min) for 14 days and examined for their vasodilator responses to ACh, an endothelium-dependent vasodilator. In arteries from untreated WT mice, ACh induced potent dilation in a concentration-dependent manner (maximal dilation at  $10^{-5}$  mol/L,  $92 \pm 2\%$ ,  $n = 9$ ; Fig. 3A). Chronic Ang II treatment caused a downward displacement in the concentration–response curve (dilation at  $10^{-5}$  mol/L,  $71 \pm 5\%$ ,  $n = 7$ ,  $P < 0.05$  vs. untreated). The pEC<sub>50</sub> values were not significantly different in control and Ang II-treated WT animals ( $6.4 \pm 0.3$  and  $6.2 \pm 0.2$ , respectively). There were no significant differences in passive diameters ( $179 \pm 8 \mu\text{m}$  in untreated and  $187 \pm 4 \mu\text{m}$  in Ang II-treated) or U46619-induced constriction (percentage constriction,  $41 \pm 2\%$  in untreated and  $45 \pm 2\%$  in Ang II-treated). As we reported previously using the same knockout mouse line (Zhang et al. 2009), ACh-induced dilation was shifted downward in TRPV4<sup>-/-</sup> mice (Fig. 3B) compared with WT control (Fig. 3A), with maximal dilation at  $10^{-5}$  mol/L of  $69 \pm 9\%$  ( $n = 5$ ,  $P < 0.05$  vs. WT control). Surprisingly, chronic treatment with Ang II did not affect ACh dilation in TRPV4<sup>-/-</sup> mice (dilation at  $10^{-5}$  mol/L,  $72 \pm 4\%$ ,  $n = 5$ ). The pEC<sub>50</sub> values were similar in control and Ang II-treated TRPV4<sup>-/-</sup> animals ( $6.2 \pm 0.2$  and  $6.3 \pm 0.1$ , respectively). No significant differences were noted in passive

diameters ( $189 \pm 8 \mu\text{m}$  in untreated and  $187 \pm 4 \mu\text{m}$  in Ang II-treated) or U46619-induced constriction (percentage constriction,  $40 \pm 3\%$  in untreated and  $42 \pm 2\%$  in Ang II-treated). Vasodilation to GSK1016790A, a potent TRPV4 agonist and endothelium-dependent vasodilator (Willette et al. 2008; Mendoza et al. 2010), was also markedly reduced in Ang II-treated WT mice (at  $10^{-8}$  mol/L,  $14 \pm 5\%$  vs.  $77 \pm 7\%$  in untreated,  $n = 7$ ,  $P < 0.05$ ; Fig. 3C). GSK1016790A did not dilate mesenteric arteries from TRPV4<sup>-/-</sup> mice (Fig. 3D), confirming the specificity of this compound for TRPV4 channels. Together, these results indicate that TRPV4 is involved in Ang II-induced impairment of endothelium-dependent vasodilation.

We next determined the main vasodilator factor(s) (NO vs. EDHFs) that are affected by Ang II infusion. In U46619-precontracted mesenteric arteries from untreated WT mice, L-NAME markedly inhibited ACh-induced dilation, an effect that was more pronounced at lower (probably more physiological) concentrations of ACh (Kawada et al. 1985; Shinoue et al. 2005) (dilation at  $10^{-7}$  mol/L,  $11 \pm 4\%$  vs.  $41 \pm 6\%$  in controls,  $n = 5$ ,  $P < 0.05$ ; Fig. 4A), confirming previous results that NO is a major vasodilator factor in this isolated vessel preparation (Chataigneau et al. 1999; Zhang et al. 2009). The effect of L-NAME plus the cyclooxygenase inhibitor indomethacin was not examined in



**Figure 3.** Effect of angiotensin II on acetylcholine- and TRPV4 agonist-induced dilation in small mesenteric arteries from wild-type (WT) and TRPV4<sup>-/-</sup> mice. Animals were chronically infused with angiotensin II (600 ng/kg per minute) for 14 days. Vasodilator responses to acetylcholine, an endothelium-dependent vasodilator, were performed on isolated U-46619-precontracted arteries from untreated (control) and angiotensin II (Ang II)-treated WT (A) and TRPV4<sup>-/-</sup> (B) mice. Vasodilator responses to GSK1016790A, a selective TRPV4 agonist, were examined in parallel arteries from same untreated and Ang II-treated WT (C) and TRPV4<sup>-/-</sup> (D) mice.  $n = 5-9$  animals per group. \* $P < 0.05$  vs. control.

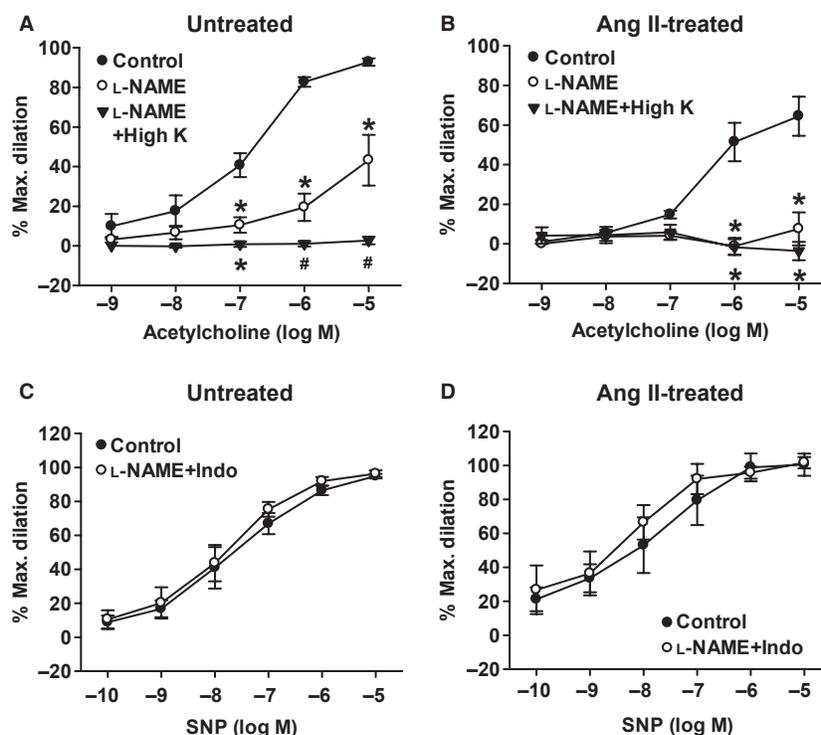
this study, as our previous studies have found that L-NAME in combination with indomethacin does not further reduce ACh-induced dilation (Zhang et al. 2009). ACh-induced dilation was abolished by the combination of L-NAME and high K, indicating that EDHF mediates the remaining dilation (Fig. 4A). Compared with untreated WT mice (Fig. 4A), the L-NAME-sensitive component of vasodilation was significantly reduced after chronic Ang II infusion, in particular at lower concentrations of ACh (dilation at  $10^{-7}$  mol/L,  $4 \pm 2\%$  in the presence vs.  $15 \pm 2\%$  in the absence of L-NAME,  $n = 5$ ; Fig. 4B), suggesting that reduced NO is mainly responsible for the impaired dilation to lower concentrations of ACh. Endothelium-independent dilation in response to the NO donor SNP ( $10^{-10}$ – $10^{-5}$  mol/L) was similar in untreated and Ang II-treated groups (Fig. 4C and D), indicating that Ang II does not affect NO sensitivity.

### TRPV4 expression and activity in Ang II-induced endothelial dysfunction

To determine whether Ang II impairs endothelium-dependent dilation by reducing TRPV4 expression,

TRPV4 protein expression was examined in mesenteric arteries freshly isolated from untreated and Ang II-treated animals. Ang II did not significantly affect total cellular protein expression levels of TRPV4 (Fig. 5A), nor did it alter the protein expression of eNOS (Fig. 5B). Given that Ang II had little effect on the protein expression of TRPV4 channels, we next tested whether Ang II-induced endothelial dysfunction is associated with reduced TRPV4 channel activity. TRPV4-mediated  $Ca^{2+}$  response was examined in endothelial cells in situ of mesenteric arteries freshly isolated from WT mice with or without Ang II infusion. As shown in Figure 6, the TRPV4 agonist GSK1016790A (10 and 30 nmol/L) induced a rapid concentration-dependent  $[Ca^{2+}]_i$  increase in endothelial cells of untreated animals, with delta changes of  $F_{340}/F_{380}$   $0.13 \pm 0.02$  and  $0.28 \pm 0.01$ , respectively. Similar to TRPV4 protein expression, this GSK1016790A-induced  $Ca^{2+}$  response was not altered by Ang II treatment ( $\Delta F_{340}/F_{380}$ ,  $0.13 \pm 0.06$  and  $0.32 \pm 0.09$  in response to 10 and 30 nmol/L, respectively;  $n = 4-5$ ).

We further examined the effect of Ang II on the cell surface protein expression and function of TRPV4 channels using cultured endothelial cells. It is difficult to



**Figure 4.** Effect of angiotensin II on endothelium- and smooth muscle-mediated dilation in small mesenteric arteries from wild-type mice. Animals were chronically infused with angiotensin II (600 ng/kg per minute) for 14 days. Acetylcholine-induced dilation, in the absence or presence of L-NAME (100  $\mu\text{mol/L}$ ) or L-NAME plus high K (60 mmol/L), was examined in isolated arteries from untreated (A) and angiotensin II (Ang II)-treated (B) mice. Vasodilation to the smooth muscle relaxant sodium nitroprusside (SNP), in the absence or presence of L-NAME plus indomethacin (Indo, 10  $\mu\text{mol/L}$ ), was examined in parallel arteries from same untreated (C) and Ang II-treated (D) mice.  $n = 5$  animals for each group. \* $P < 0.05$  versus control. # $P < 0.05$  versus L-NAME.

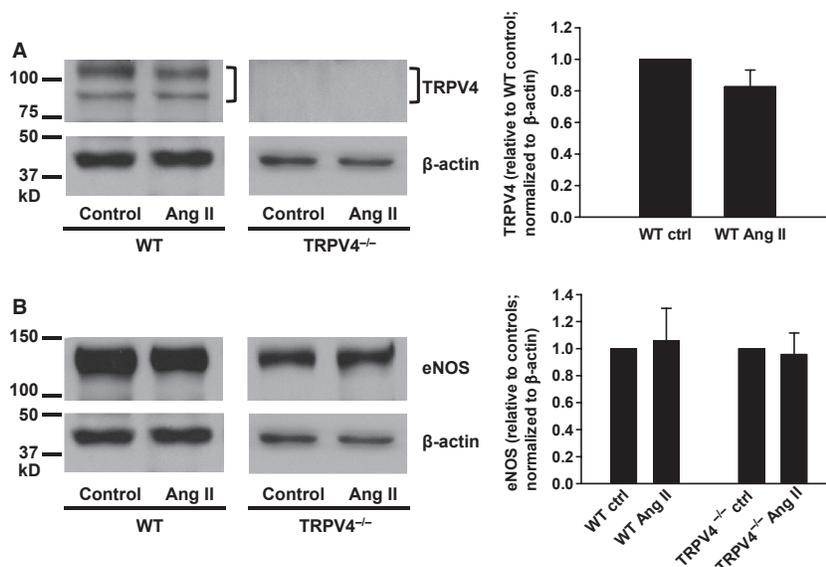
detect cell surface TRPV4 channels in isolated small mesenteric arteries and in native endothelial cells due to their low abundance on the plasma membrane, therefore hTRPV4-overexpressing HCAECs (Zheng et al. 2013) were used for these experiments. As shown in Figure 7A, Ang II treatment (500 nmol/L for 1 or 24 h) did not alter either the total or cell surface TRPV4 expression in HCAECs. In contrast, the TRPV4 agonist  $4\alpha$ -PDD (3  $\mu\text{mol/L}$ )-induced  $[\text{Ca}^{2+}]_i$  increase was markedly reduced in native nontransfected HCAECs after 24-h treatment with Ang II ( $F_{340}/F_{380}$  ratio,  $1.0 \pm 0.1$  vs.  $1.9 \pm 0.2$  of control; Fig. 7B). This  $4\alpha$ -PDD-induced response was slightly attenuated (but not statistically significant) after 1 h Ang II treatment. Ang II also inhibited GSK1016790A-induced  $\text{Ca}^{2+}$  response in non-transfected HCAECs (data not shown).

## Discussion

This research aimed at understanding the role of endothelial TRPV4 channels in the regulation of blood pressure and endothelial function under hypertensive challenges.

The major findings of this study are as follows: (1) L-NAME but not Ang II induced a slightly greater hypertensive response in TRPV4<sup>-/-</sup> mice compared with WT controls, though TRPV4<sup>-/-</sup> mice have lower MAP under basal conditions; (2) chronic treatment of WT mice with Ang II reduced ACh- and TRPV4 agonist-induced dilation of small mesenteric arteries, but surprisingly Ang II treatment did not affect ACh dilation in TRPV4<sup>-/-</sup> mice; (3) mechanistically, Ang II did not significantly alter either TRPV4 total protein expression in mesenteric arteries or TRPV4 agonist-induced  $\text{Ca}^{2+}$  response in mesenteric endothelial cells in situ. In cultured endothelial cells, Ang II treatment had no effect on cell surface localization of TRPV4 but attenuated TRPV4-mediated  $\text{Ca}^{2+}$  response. Together, these data suggest a minor protective role for TRPV4 in L-NAME-induced hypertension. In contrast, TRPV4 does not seem to contribute to blood pressure regulation in Ang II-induced hypertension, but may be importantly involved in Ang II-induced endothelial dysfunction.

Studies from both animal models and humans have demonstrated a strong association between endothelial

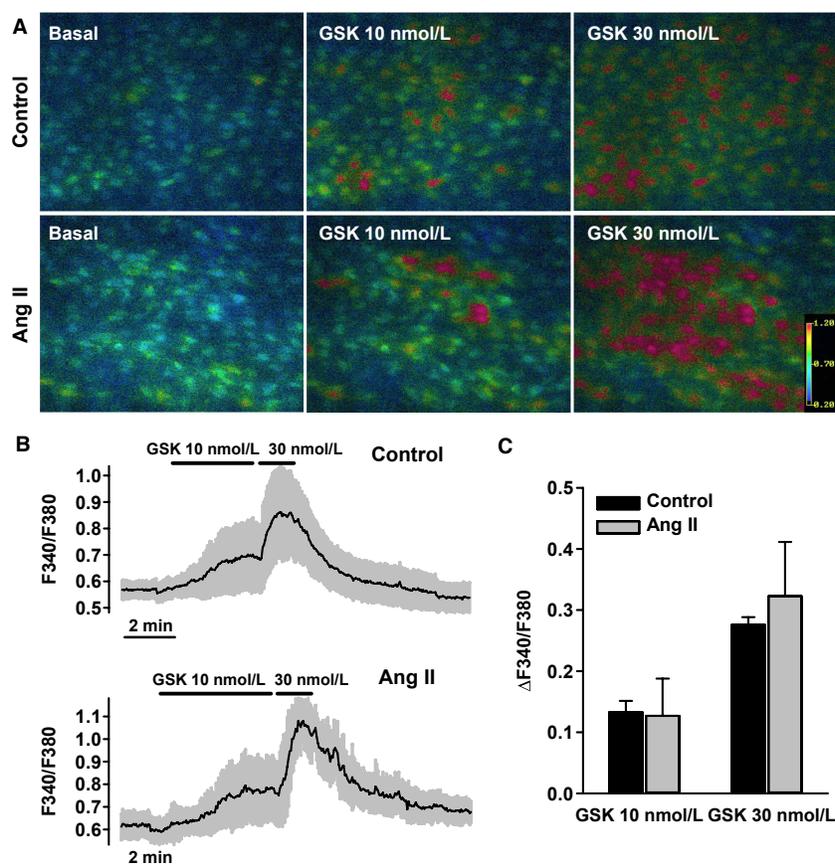


**Figure 5.** Protein expression of TRPV4 and endothelial NO synthase (eNOS) in small mesenteric arteries from wild-type (WT) and TRPV4<sup>-/-</sup> mice treated with angiotensin II (Ang II). Animals were chronically infused with angiotensin II (600 ng/kg per minute) for 14 days. Mesenteric arteries were isolated and examined for TRPV4 (A) and eNOS (B) protein expression by Western blotting. Left, representative blots; right, summarized data (normalized to  $\beta$ -actin loading controls).  $n = 6$ –9 animals per treatment group.

dysfunction (or reduced endothelium-dependent dilation) and hypertension, although whether endothelial dysfunction is a cause or an effect of hypertension remains poorly understood (Dharmashankar and Widlansky 2010; Quyyumi and Patel 2010). By measuring TRPV4 agonist-induced depressor effects and changes of TRPV4 protein expression in hypertensive rat models, Gao *et al.* (2009) and Gao and Wang (2010) provided evidence that altered protein expression and function of vascular TRPV4 may be associated with salt-induced hypertension. Using a genetic knockout model and a tail-cuff method to measure blood pressure, Earley and colleagues reported a slightly higher blood pressure after NOS inhibition with *N*<sup>ω</sup>-nitro-L-arginine in TRPV4<sup>-/-</sup> mice compared with WT controls (Earley *et al.* 2009) and thus proposed that endothelial TRPV4 channels may serve as a backup protective mechanism in NOS inhibitor-induced hypertension. In this study, we measured 24-h blood pressure responses in another line of TRPV4<sup>-/-</sup> mice given two different hypertensive challenges. Consistent with previous results (Earley *et al.* 2009), the percent increase in MAP in response to oral administration of L-NAME (another NOS inhibitor) was larger in TRPV4<sup>-/-</sup> animals. However, a greater MAP response to Ang II infusion was not apparent in TRPV4<sup>-/-</sup> mice compared with WT controls, which may be partially due to impaired TRPV4 function in Ang II-treated WT controls (Fig. 3). It remains to be determined whether loss of TRPV4 function occurs in and/or contributes to certain types of hypertension in

humans. Besides endothelium-mediated dilation, other potential mechanisms by which TRPV4 contributes to the regulation of blood pressure *in vivo* remains to be clarified. For example, L-NAME-induced HR reduction was less in TRPV4<sup>-/-</sup> mice, suggesting that TRPV4 channels may be involved in the regulation of baroreflex responses.

An impairment of NO- and/or EDHF-mediated endothelium-mediated dilation is frequently observed under various pathophysiological conditions or in diseases such as CAD, diabetes, and hypertension (Félétou and Vanhoutte 2004; Vanhoutte *et al.* 2009). As an important determinant of vascular disease, Ang II increases vascular ROS production and reduces NO-mediated vasodilation (Rajagopalan *et al.* 1996; Jung *et al.* 2003). The precise molecular mechanisms by which Ang II alters intracellular signaling components leading to endothelial dysfunction remain largely unclear. In this study, ACh-induced vasodilation was markedly reduced in Ang II-treated WT mice mesenteric arteries, which is consistent with previous findings from others (Viridis *et al.* 2004; De Ciuceis *et al.* 2005; Gongora *et al.* 2006). The impaired dilation to ACh, in particular at lower concentrations of ACh, was mainly due to reduced endothelial NO rather than changes in NO sensitivity of smooth muscle cells. Interestingly, Ang II also significantly reduced the dilation to the TRPV4 agonist GSK1016790A in WT mesenteric arteries, the dilation of which also involves endothelial NO under basal conditions (Mendoza *et al.* 2010). These results indicate that, similar to its effect on receptor ago-

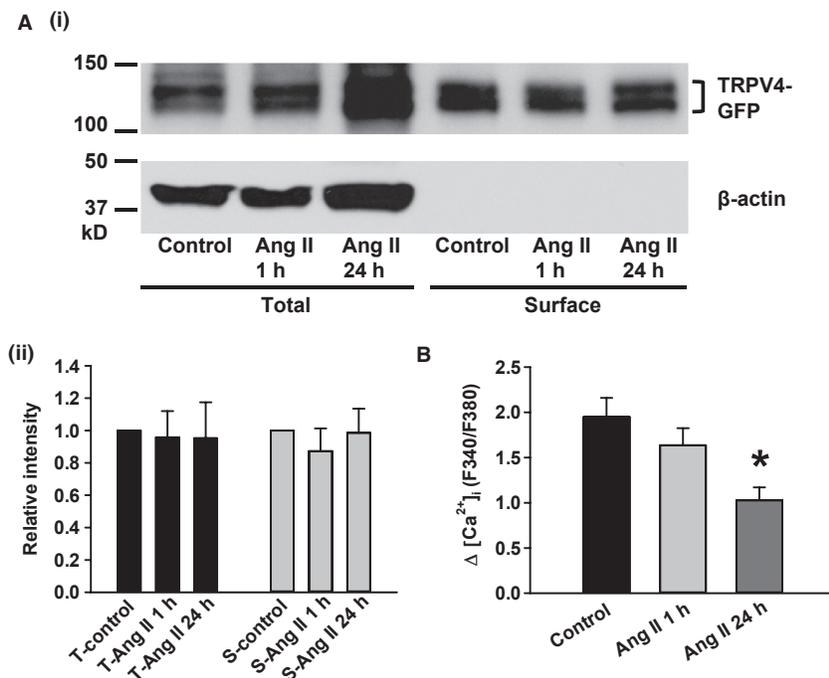


**Figure 6.** TRPV4-mediated  $\text{Ca}^{2+}$  response in endothelial cells in situ of mesenteric arteries isolated from wild-type mice with or without angiotensin II treatment. Animals were chronically infused with angiotensin II (600 ng/kg per minute) for 14 days. Mesenteric arteries were freshly isolated and endothelial cells were loaded with fura-2 AM for intracellular  $\text{Ca}^{2+}$  imaging. (A) Representative images of endothelial  $\text{Ca}^{2+}$  responses to GSK1016790A (10 and 30 nmol/L), a TRPV4-specific agonist, in untreated and Ang II-treated mice. (B) Representative traces of endothelial  $\text{Ca}^{2+}$  responses to GSK1016790A in untreated and Ang II-treated mice. The black line denotes mean ratio of the fluorescence intensity at 340- versus 380-nm excitation (F340/F380), and the gray area indicates one standard deviation. (C) Summarized data of endothelial  $[\text{Ca}^{2+}]_i$  increase. Data represent mean  $\pm$  SEM of 4–5 animals for each group, with 20–30 endothelial cells analyzed per each vessel.

nist ACh, Ang II treatment induces a functional impairment of TRPV4-mediated dilation in mesenteric arteries. Given that TRPV4-dependent  $\text{Ca}^{2+}$  entry has been implicated in ACh-induced dilation in this vascular bed (Earley et al. 2009; Zhang et al. 2009; Adapala et al. 2011; Sonkusare et al. 2012), these results indicate that reduced TRPV4-mediated dilation may represent a previously unrecognized endothelial component contributing to Ang II-induced endothelial dysfunction. Ang II did not affect ACh-induced dilation in TRPV4<sup>-/-</sup> mice, a largely unexpected finding that further implies a potential role of TRPV4 in vascular effects of Ang II (more discussion below).

This study also provides some mechanistic insights into the role of TRPV4 channels in Ang II-induced impairment of endothelium-dependent vasodilation. Compared with arteries from untreated animals, TRPV4 protein

expression and TRPV4-mediated endothelial  $\text{Ca}^{2+}$  response were similar in freshly isolated mesenteric arteries from Ang II-treated mice. In addition, chronic Ang II infusion did not affect eNOS protein expression in WT or TRPV4<sup>-/-</sup> mice. These results indicate that the impaired dilation in response to ACh and GSK1016790A following Ang II treatment is unlikely due to altered protein expression of TRPV4 or eNOS, or reduced  $\text{Ca}^{2+}$  entry via TRPV4 in endothelial cells. Instead, Ang II may affect other signaling events downstream of TRPV4 and eNOS activation in endothelial cells, which consequently leads to the impaired endothelium-dependent dilation. Further studies are required to test these possibilities. Using vascular smooth muscle cells and overexpression cell systems, Shukla et al. (2010) recently reported that  $\beta$ -arrestin 1 recruited by Ang II receptor activation reduces cell surface protein expression of TRPV4 channels



**Figure 7.** Effect of angiotensin II (Ang II) on TRPV4 protein expression and TRPV4-mediated  $Ca^{2+}$  response in endothelial cells. (A) Western blot analysis of total and cell surface protein expression of TRPV4 in hTRPV4-overexpressing human coronary artery endothelial cells (HCAECs) incubated with Ang II (500 nmol/L) for 1 or 24 h. (i), representative blots; (ii), summarized data (normalized to  $\beta$ -actin for total protein) of 3 independent experiments. (B) Fura-2  $Ca^{2+}$  assay of TRPV4-mediated  $Ca^{2+}$  response to TRPV4 agonist 4 $\alpha$ -PDD (3  $\mu$ mol/L) in native HCAECs treated with Ang II (500 nmol/L) for 1 or 24 h. The data represent mean  $\pm$  SEM of  $\geq 60$  cells analyzed in three independent experiments. \* $P < 0.05$  vs. control. T, total protein; S, surface protein.

and TRPV4-mediated  $Ca^{2+}$  response by enhancing channel internalization (without degradation). In cultured endothelial cells (HCAECs), Ang II treatment for 1 and 24 h at a similar concentration used in previous studies (Shukla *et al.* 2010) did not affect either the total or cell surface TRPV4 expression. However, Ang II treatment for 24 h significantly reduced TRPV4 agonist-induced  $[Ca^{2+}]_i$  increase in HCAECs, indicating an impaired intracellular signaling leading to TRPV4 activation. The reasons for these discrepancies are unclear, but could be due to difference in the type of tissues/cells used or in experimental conditions (in vivo vs. in vitro). In addition, a concentration-related differential effect of Ang II on TRPV4 expression and function cannot be excluded. It has been reported previously that Ang II induces type I Ang II receptor signaling, desensitization or internalization in a concentration-dependent manner (Tang *et al.* 1995).

Because Ang II did not affect ACh-induced dilation in TRPV4 $^{-/-}$  animals, it is possible that Ang II may conversely activate TRPV4 channels in endothelial cells to elicit subsequent signaling events such as increased ROS production and reduced NO bioavailability, an effect that may be more important with lower or sub-

pressor doses of Ang II. There is evidence that upregulation of Ang II signaling causes endothelial dysfunction independent of blood pressure responses or in the absence of blood pressure elevation (Liu *et al.* 2003; Marchesi *et al.* 2013). It is, therefore, of interest to determine in future studies whether subpressor doses of Ang II induce endothelial dysfunction in a TRPV4-dependent manner.

Due to technical difficulty, we were unable to use the biotinylation technique to directly study the cell surface expression of TRPV4 channels in endothelial cells of mesenteric arteries from Ang II-treated animals. An alternative approach using antibodies against extracellular epitopes of TRPV4 channels might be considered, but the only antibody that is currently available did not detect TRPV4 protein in mouse vessels (data not shown).

## Perspectives

As a  $Ca^{2+}$  entry channel expressed in endothelial cells of multiple vascular beds from several species including humans, TRPV4 has been implicated in endothelium-mediated dilation in response to receptor agonists as

well as to shear stress (Köhler et al. 2006; Hartmannsgruber et al. 2007; Loot et al. 2008; Earley et al. 2009; Zhang et al. 2009; Mendoza et al. 2010; Adapala et al. 2011; Bubolz et al. 2012; Rath et al. 2012; Sonkusare et al. 2012; Ma et al. 2013; Zheng et al. 2013). Using a streptozotocin-induced diabetic rat model, Ma et al. (2013) have recently reported that a reduced TRPV4-mediated TRPV4 signaling may be an underlying mechanism responsible for the impaired EDHF-dependent vasodilation in diabetes. In this study, we found that L-NAME increases blood pressure to a greater extent in TRPV4<sup>-/-</sup> mice compared to WT controls, but paradoxically TRPV4<sup>-/-</sup> mice are protected from Ang II-induced endothelial dysfunction in small resistance arteries. These results suggest potential divergent functions of TRPV4 in vascular diseases. Interestingly, two recent studies have shown that TRPV4-deficient mice or WT mice treated with TRPV4 antagonists are also protected from diet-induced obesity, adipose inflammation, and insulin resistance (Kusudo et al. 2012; Ye et al. 2012). In the human coronary microcirculation from subjects with CAD, data from our laboratory indicate that TRPV4-mediated Ca<sup>2+</sup> entry serves as an important signaling event leading to flow-induced release of H<sub>2</sub>O<sub>2</sub> (Bubolz et al. 2012; Zheng et al. 2013), a ROS that mediates continued dilatory response during disease in the absence of traditional endothelial relaxing factors (i.e., NO and PGI<sub>2</sub>) but also has atherogenic properties potentially contributing to CAD and other cardiovascular diseases (Miura et al. 2003; Liu et al. 2011; Zhang et al. 2012). It is, therefore, of substantial interest to determine in future studies the precise roles of TRPV4 in vascular diseases, especially in humans.

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## Conflict of Interest

None declared.

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